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ORIGINAL PAPER

# Apoptosis, proliferation, and cell size in seasonal changes of body and organ weight in male bank voles *Myodes glareolus*

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**Abstract** Adult male bank voles undergo the body and organ regression before winter, and in early spring, they resume the growth and reproductive processes. The aim of the present study was to determine whether the seasonal changes of body and organ weight in these animals depend on changes in the number of cells or their size. To study an autumnal regression, wild adult males captured in August were exposed to short photoperiod for 0, 4, and 8 weeks, while to study a spring resumption, overwintered males caught in March were exposed to long photoperiod for 0, 1, and 4 weeks. Apoptosis, proliferation, and cell size in the skeletal muscles, liver, and testes were examined. The study revealed that the seasonal changes of testes weight were associated with changes in the number of testicular cells. On the contrary, the changes in size of skeletal myocytes and hepatocytes appeared to be responsible for the seasonal changes of body (muscle) and liver weights in these rodents.

**Keywords** Apoptosis · Cell proliferation · Cell size · Liver · Muscle · Testes

## Introduction

The bank vole *Myodes glareolus* is a winter-active rodent and, like many small mammals, shows a winter decline in body weight; it has been shown that male bank voles during winter are even 40 % lighter than those from spring and summer (Klaus et al. 1988; Włostowski et al. 1988; Bonda-Ostaszewska et al. 2012). The body mass decline is thought to be a mechanism by which the animals offset winter increased energy demands (Lovegrove 2005; McNab 2010).

Seasonal changes in body mass of bank voles and other small mammals from the temperate zone are under control of photoperiod (Dark et al. 1983; Bartness et al. 2002; Bonda-Ostaszewska et al. 2012), and in some species, the changes in white and brown adipose tissues are thought to contribute significantly to the changes in body weight (Bartness et al. 2002). However, the bank voles belong to those species which exhibit no seasonal changes in fat mass (Klaus et al. 1988; Bonda-Ostaszewska et al. 2012), and the changes in weight of other organs such as liver, kidneys, and testes contribute only slightly (about 10 %) to the changes of body weight (Włostowski et al. 1988, 2009). Thus, other tissues, e.g., skeletal muscles which account for 24–61 % of total body mass (Raichlen et al. 2010), are probably responsible for the seasonal changes of body weight in these animals. The bank voles enter the winter season as young immature individuals and as adult animals which underwent the body and organ regression in autumn. After winter, they resume the growth and reproductive processes (Włostowski et al. 1988, 2009). So far, however, the mechanism of an autumnal regression of the body and organs as well as a spring resumption of the growth has not been elucidated.

It is known that an organ weight is determined by the number of cells and/or their size. The number of cells is related to the rate of their proliferation and/or programmed cell death (apoptosis). Apoptosis allows to eliminate excessive or unnecessary cells without negative consequences for the remaining cells, and it is involved in the control of cell differentiation and remodeling of embryonic tissues (Fuchs and Steller 2011), the involution of some organs such as the mammary gland and thymus (Hojilla et al. 2011; Linkova et al. 2011), and aging (Zhang et al. 2003). Moreover, previous studies on mammals

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sensitive to changes in daylength showed that apoptosis is responsible for testicular regression (Young et al. 1999; Young and Nelson 2001; Morales et al. 2002; Strbenc et al. 2003; Luaces et al. 2014). However, it is unknown whether this process is involved in the regression of other organs and tissues such as the liver and skeletal muscles.

The present work was designed to find out whether the seasonal changes of body (muscle), liver, and testes weights in bank voles depend on changes in the number of cells and/or their size. In particular, apoptosis and proliferation in the testes, liver, and skeletal muscles during an autumnal regression and spring resumption were determined. Concurrently, cellular size was measured.

## Materials and methods

### Animals and experimental design

To study seasonal changes, thirty-one male bank voles were captured from April 2006 to February 2007 in live traps in the Knyszyn Old Forest near Białystok (northeastern Poland). They were transported to the laboratory on the same day, weighed and euthanized. The animals were assigned into four groups according to the month of capture: (1) spring: April–May, (2) summer: June–August, (3) autumn: September–November, and (4) winter: December–February.

To study an autumnal regression, a group of adult males (3–4 months old) captured in August 2006 was exposed to short photoperiod (8 h light/16 h dark) for 0, 4, and 8 weeks under laboratory conditions. Another group of animals caught in March 2007 (at least 7 months old) was exposed to long photoperiod (16 h light/8 h dark) for 0, 1, and 4 weeks to examine a spring resumption of the growth. The animals were housed individually in stainless steel cages (40×25×15 cm) lined with peat as an absorptive material and hay in the nest compartment at a constant temperature (19±1 °C) and 50–70 % relative humidity. They received ad libitum tap water and whole wheat grains; in addition, an apple was offered to all animals (Włostowski et al. 1996).

### Histological procedure and cell size measurement

The animals were euthanized by cervical dislocation. The testes, liver, and skeletal muscles associated with the femur were removed and weighed. A part of the liver and muscle was fixed in 4 % formaldehyde, while the testes were fixed in Bouin's fluid. The tissues were dehydrated in ethanol, permeabilized in xylene, embedded in paraffin, and cut into 5-μm sections (Leica microtome). The sections were then deparaffinized in xylene, rehydrated in graded ethanol series and distilled water, and stained with hematoxylin and eosin or used for apoptosis and cell proliferation detections. The light microscope (Leica) and color digital video camera were used during examination. The surface area ($\mu m^2$) of hepatocytes and myocyte cross-section ($n$=50 cells/vole) was measured using MultiScanBase v.14 (Computer Scanning System CSS). Testicular cell size could not be measured because the borderline was not seen clearly; instead, a number of testicular cells (the sum of spermatogonia, spermatocytes, and spermatids per seminiferous tubule cross-section) were estimated.

### In situ apoptosis detection

Apoptosis in the testes, liver, and muscles was demonstrated by the TdT-mediated dUTP-fluorescein nick end labeling (TUNEL) assay using "In Situ Cell Death Detection Kit, AP" (Roche Diagnostics, Manheim, Germany). Briefly, sections were permeabilized in 0.1 % Triton X-100/0.1 % sodium citrate, and fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase (TdT) enzyme were applied for 60 min at 37 °C. Subsequently, the sections were washed with phosphate buffered saline (PBS) and treated with alkaline phosphatase-conjugated anti-fluorescein antibody for 30 min at 37 °C. Next, they were treated with substrate solution (NBT/BCIP) for 10 min in the dark. Apoptosis in the testes was expressed as the number of TUNEL-positive cells per seminiferous tubule, while in the liver and muscle, as the number of TUNEL-positive cells per microscopic field (objective ×40).

**Table 1** Body and testes weights, testicular apoptosis, and myocyte size in the bank voles caught in different seasons

| Season (vole status) | $n$ | Body weight (g) | Myocyte cross-sectional area ($\mu m^2$) | Testes weight (g) | Apoptosis in testes (apoptotic cells/seminiferous tubule) |
|---|---|---|---|---|---|
| Spring (mature) | 5 | $21.3\pm2.5^{a}$ | $670\pm40^{a}$ | $0.818\pm0.178^{a}$ | $0.03\pm0.01^{a}$ |
| Summer (mature) | 8 | $22.5\pm2.1^{a}$ | $730\pm52^{a}$ | $0.757\pm0.067^{a}$ | $0.03\pm0.01^{a}$ |
| Autumn (in regression) | 3 | $17.0\pm2.6^{b}$ | $480\pm50^{b}$ | $0.149\pm0.050^{b}$ | $0.36\pm0.20^{b}$ |
| Autumn (immature) | 7 | $14.0\pm1.9^{b}$ | $400\pm35^{b}$ | $0.040\pm0.005^{c}$ | $0.04\pm0.01^{a}$ |
| Winter (immature) | 8 | $13.9\pm2.3^{b}$ | $410\pm30^{b}$ | $0.032\pm0.001^{c}$ | $0.03\pm0.02^{a}$ |

Values represent mean±SD. Means in the same column marked with different superscript letters are significantly different ($p$<0.05)

**Table 2** Liver weight, hepatocyte size, and apoptosis in the liver of bank voles caught in different seasons

| Season (vole status) | *n* | Liver weight (g) | Hepatocyte surface area (μm$^2$) | Apoptotic cells/ microscopic field |
|---|---|---|---|---|
| Spring (mature) | 5 | 1.25±0.13$^a$ | 236±14$^a$ | 0.20±0.15$^a$ |
| Summer (mature) | 8 | 1.32±0.19$^a$ | 280±48$^b$ | 0.15±0.12$^a$ |
| Autumn (in regression) | 3 | 0.85±0.11$^b$ | 194±25$^c$ | 0.25±0.11$^a$ |
| Autumn (immature) | 7 | 0.75±0.13$^b$ | 180±29$^c$ | 0.18±0.10$^a$ |
| Winter (immature) | 8 | 0.81±0.11$^b$ | 170±24$^c$ | 0.25±0.18$^a$ |

Values represent mean±SD. Means in the same column marked with different superscript letters are significantly different ($p<0.05$)

### In situ cell proliferation detection

Cell proliferation in the testes, liver, and muscle was demonstrated by the BrdU immunohistochemistry assay using "Cell Proliferation Kit" (Amersham, UK). BrdU (5-bromo-2′-deoxyuridine) is a synthetic nucleoside that is an analogue of thymidine and can be incorporated into replicating DNA and subsequently localized using a specific monoclonal antibody. Bank voles were injected i.p. with a single dose (1 ml/100 g of body weight) of BrdU aqueous solution (3 mg/ml) 2 h before sacrificing. The obtained sections were treated with a reconstituted nuclease and anti-5′-bromo-2′-deoxiuridine monoclonal antibody for 60 min at room temperature. Then, the sections were washed with PBS and incubated for 30 min with peroxidase-conjugated antibody to mouse $IgG_{2a}$. The antibody-peroxidase complex was developed with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide, giving blue-black staining at sites of BrdU incorporation. Proliferation was expressed as the number of BrdU-labeled cells per seminiferous tubule or per microscopic field (objective ×40).

### Statistical analysis

The data were expressed as mean±SD. The values were analyzed by nonparametric Kruskal-Wallis ANOVA with Mann-Whitney *U* test with the Bonferroni correction (IBM SPSS Statistics 21). Differences at $p<0.05$ were considered statistically significant.

## Results

The mean body, liver, and testes weights of male bank voles caught in autumn and winter were significantly smaller ($p<0.01$) than those of animals living in spring and summer, but no seasonal changes in the rate of apoptosis in the muscle and liver were found; only males being in sexual regression exhibited a higher rate of this process in the testes (Tables 1 and 2). However, seasonal changes in the size of skeletal myocytes and hepatocytes followed a pattern similar to that of the body and liver weights, respectively (Tables 1 and 2).

**Table 3** Changes in the body and organ weights, the rate of apoptosis and proliferation, and the size or number of cells in the bank vole during an autumnal regression

| | Short photoperiod exposure time (weeks) | | |
|---|---|---|---|
| | 0 | 4 | 8 |
| Body weight (g) | 23.0±1.4$^a$ | 18.1±0.7$^b$ | 16.5±0.9$^b$ |
| Myocyte cross-sectional area (μm$^2$) | 890±35$^a$ | 578±35$^b$ | 474±30$^b$ |
| Liver weight (g) | 1.00±0.09$^a$ | 0.71±0.08$^b$ | 0.60±0.04$^b$ |
| Hepatocyte surface area (μm$^2$) | 270±42$^a$ | 170±25$^b$ | 160±29$^b$ |
| Apoptotic cells/microscopic field | 0.12±0.05$^a$ | 0.10±0.01$^a$ | 0.15±0.13$^a$ |
| BrdU-labeled hepatocytes/field | 0.15±0.09$^a$ | 0.13±0.05$^a$ | 0.17±0.08$^a$ |
| Testes weight (g) | 0.75±0.02$^a$ | 0.24±0.02$^b$ | 0.05±0.01$^c$ |
| Apoptotic cells/seminiferous tubule | 0.06±0.01$^a$ | 0.47±0.10$^b$ | 1.28±0.48$^c$ |
| BrdU-labeled cells/seminiferous tubule | 1.95±0.12$^a$ | 0.25±0.05$^b$ | 0.20±0.04$^b$ |
| Testicular cells/seminiferous tubule | 212±36$^a$ | 78±21$^b$ | 41±5$^c$ |

Values represent mean±SD ($n=4$). The animals were caught in summer and raised under short photoperiod for 8 weeks. No apoptosis and proliferation in the skeletal muscle were observed. Means in rows marked with different superscript letters are significantly different ($p<0.05$). Also, see Figs. 1 and 2

To study in more detail the regression processes, a group of adult bank voles caught in August was exposed to short photoperiod (SP) for 8 weeks under laboratory conditions. As can be seen in Table 3, the body and liver weights decreased significantly by 21 and 29 % after 4 weeks and 28 and 40 % after 8 weeks of SP exposure, respectively; in accordance, the size of myocytes and hepatocytes decreased by 35 and 37 % after 4 weeks and 47 and 40 % after 8 weeks of the exposure. During the body (muscle) and liver regression, no changes in the rate of apoptosis and proliferation were observed, but testicular regression was accompanied by a significant rise in apoptotic cells and decline in their proliferation and number (Fig. 1, Table 3).

To examine a spring resumption of the growth of bank voles, a group of overwintered males caught in March was raised in a long photoperiod (LP) for 4 weeks under laboratory conditions (Table 4). A statistically significant ($p<0.05$) body weight gain (by 20 %) was observed after 1 week of LP exposure; after 4 weeks of the exposure, the body weight increased by 50 % and attained the level typical for adult male bank voles. These changes in the body weight as well as an increase in the liver mass were accompanied by a rise in the size of myocytes (by 41 and 95 %) and hepatocytes (by 24 and 51 %) after 1- and 4-week exposures to a long photoperiod, respectively (Table 4). Notably, no increase in the new cell generation in the liver and skeletal muscle was found; however, proliferation and cell number in the testes increased significantly during the resumption in March (Table 4).

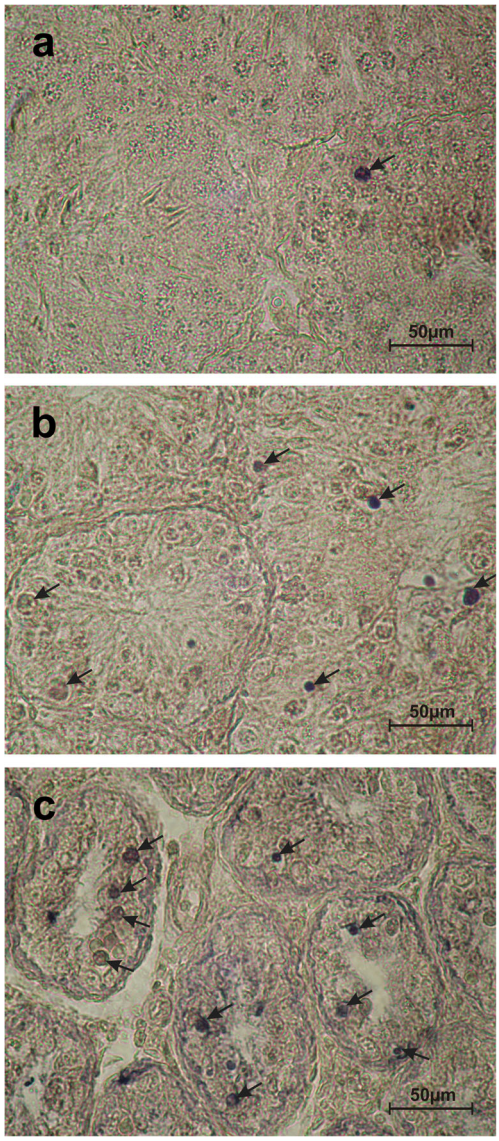


**Fig. 1** Immunohistochemical demonstration of apoptotic cells in the testes of bank voles raised under short photoperiod for 0 (**a**), 4 (**b**), and 8 (**c**) weeks. Note an increase in apoptotic cells (*arrows*) and reduction in seminiferous epithelium in the testes after 4 (**b**) and 8 (**c**) weeks. Also, see Table 3

## Discussion

The results of the present study confirmed previous observations that changes in the number of testicular cells are responsible for the seasonal changes of testes weight in small mammals sensitive to photoperiod (Young et al. 1999; Young and Nelson 2001; Morales et al. 2002; Sato et al. 2005; Pastor et al. 2011; Seco-Rovira et al. 2014). In contrast to the testes, changes in the number of myocytes and hepatocytes appeared not to significantly contribute to the seasonal changes of body (muscle) and liver weights in the bank vole. This is evidenced by very low rate of apoptosis during the autumnal regression of muscle and liver as well as of the new cell generation during the spring resumption of the growth (Tables 3 and 4). These results are in agreement with literature data which indicate that there is generally no recruitment of new fibers in skeletal muscle following birth or new fiber formation occurs only during early postnatal development (Goldspink 1972; Tamaki et al. 2002), and apoptosis of muscle is only known during disease and aging (Agusti et al. 2002; Dirks and Leeuwenburgh 2002). Likewise, proliferation and apoptosis are not responsible for the enlargement and regression of liver mass in adult mice (Bursch et al. 2005). Thus, it appears that other processes are involved in the seasonal changes of body (muscle) and liver weights in the bank vole.

Our work showed that changes in the size of myocytes and hepatocytes may contribute significantly to the seasonal changes of body (muscle) and liver weights in bank voles. Indeed, during the autumnal regression, the size of myocytes and hepatocytes decreased by up to 40–47 % in accord with the body and liver weights decline (28–40 %), while during the spring resumption their size increased by 50–95 %. These data suggest that seasonal changes in body (muscle) and liver weights of bank voles primarily depend on cell size changes.

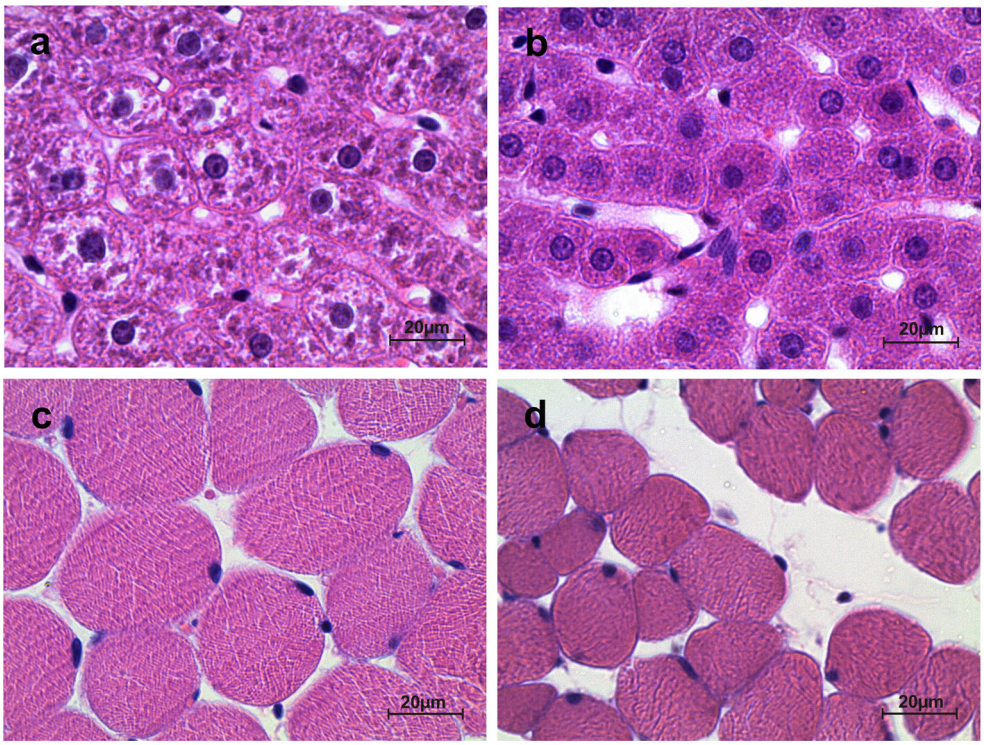


**Fig. 2** Representative photomicrographs of the liver (**a**, **b**) and skeletal muscle (**c**, **d**) sections in the bank vole raised under short photoperiod for 0 (**a**, **c**) and 8 (**b**, **d**) weeks. Note a decrease in hepatocyte and myocyte sizes after 8 weeks. Also, see Table 3

Several studies revealed that seasonal changes in body and organ weights of bank voles and other small mammals remain under control of the photoperiod (Dark et al. 1983; Bartness et al. 2002; Peacock et al. 2004; Włostowski et al. 2004; Król et al. 2005). It is reasonable to conclude that the effect of photoperiod on myocyte and hepatocyte cell size is also mediated through the short-photoperiod mediator melatonin (Bartness et al. 2002; Włostowski et al. 2005).

It is well known that photoperiod exerts its effects through neural regulation of the pineal-hypothalamus-pituitary-gonadal axis (Bartness at al. 2002); a short photoperiod or long duration of melatonin secretion by the pineal gland triggers testicular regression and reduction in androgen secretion, while a long photoperiod or short duration of melatonin secretion has opposite effects (Tables 3 and 4; Tähkä et al. 1997). Additionally, it has been demonstrated that testicular

**Table 4** Changes in the body and organ weights, the rate of cell proliferation (BrdU) and apoptosis, and the size or number of cells in the bank vole during spring resumption

| | February | Long photoperiod exposure time (weeks) | | |
|---|---|---|---|---|
| | | March—0 (start of experiment) | 1 | 4 |
| Body weight (g) | $14.5\pm1.0^{a}$ | $16.7\pm0.9^{a}$ | $20.0\pm0.8^{b}$ | $25.5\pm1.4^{c}$ |
| Myocyte cross-sectional area ($\mu m^2$) | $430\pm30^{a}$ | $460\pm32^{a}$ | $650\pm40^{b}$ | $900\pm60^{c}$ |
| Liver weight (g) | $0.79\pm0.08^{a}$ | $0.80\pm0.05^{a}$ | $0.95\pm0.10^{ab}$ | $1.10\pm0.13^{b}$ |
| Hepatocyte surface area ($\mu m^2$) | $180\pm20^{a}$ | $185\pm16^{a}$ | $230\pm20^{ab}$ | $280\pm30^{b}$ |
| BrdU-labeled hepatocytes/field | $0.15\pm0.07^{a}$ | $0.30\pm0.15^{a}$ | $0.45\pm0.20^{a}$ | $0.35\pm0.25^{a}$ |
| Apoptotic cells/microscopic field | $0.16\pm0.12^{a}$ | $0.15\pm0.10^{a}$ | $0.11\pm0.08^{a}$ | $0.12\pm0.09^{a}$ |
| Testes weight (g) | $0.03\pm0.01^{a}$ | $0.34\pm0.05^{b}$ | $0.76\pm0.06^{c}$ | $0.85\pm0.01^{c}$ |
| BrdU-labeled cells/seminiferous tubule | $0.05\pm0.01^{a}$ | $2.10\pm0.10^{b}$ | $2.00\pm0.10^{b}$ | $1.50\pm0.15^{b}$ |
| Apoptotic cells/seminiferous tubule | $0.03\pm0.01^{a}$ | $0.04\pm0.01^{a}$ | $0.03\pm0.02^{a}$ | $0.04\pm0.01^{a}$ |
| Testicular cells/seminiferous tubule | $40\pm6^{a}$ | $120\pm25^{b}$ | $230\pm60^{c}$ | $294\pm58^{c}$ |

Values represent mean±SD ($n$=4). The animals were caught in February and March, and the latter ones were raised in the laboratory under long photoperiod for 4 weeks. No cell proliferation and apoptosis in the skeletal muscle were observed. Means in rows marked with different superscript letters are significantly different ($p<0.05$)

secretions promote weight gain, and their absence results in weight loss in several small mammals (Dark et al. 1983; Bartness at al. 2002). Also, the results of the present study indicate that photoperiod-induced changes in testicular weight correlate significantly with the body weight of bank voles ($r$= 0.91, $p$<0.001) (Tables 3 and 4), suggesting direct actions of androgens on myocyte size. The involvement of androgens may be the case because body weight gain of female bank voles raised in a long photoperiod is not or only slightly higher than that of females kept in a short photoperiod (Włostowski et al. 1996; Peacock et al. 2004). Since androgens are known to stimulate protein synthesis (Roy et al. 1999), it is likely that changes in the protein content may be responsible for oscillations in the size of myocytes and hepatocytes in the bank vole. Indeed, changes in cellular size have been shown to primarily result from oscillations in the protein content (Bursch et al. 2005). Still, the precise mechanism of cellular hyper-/hypotrophy in these animals remains to be determined.

In conclusion, the results of the present study showed that changes in the number of cells contributed significantly to the seasonal changes of testes, but not muscle and liver weights in the bank vole. Instead, changes in the size of skeletal myocytes and hepatocytes appeared to be responsible for the seasonal changes of body (muscle) and liver weights in these animals.

**Ethics** All experimental procedures were approved by the Local Ethical Committee (Medical University of Bialystok) and were compatible with standards of the Polish Law on Experimenting on Animals, which implements the European Communities Council Directive (86/609/EEC).

**Conflict of interest** The authors declare that they have no conflict of interest.



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